Apoptosis and Autophagy induction in T98G cells (glioblastoma) with Concanavalin A

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BY

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CERTIFICATE

This is to certify that the thesis entitled "**Apoptosis and autophagy induction in T98G cells with Concanavalin A**" which is being submitted by Ms Tanmayee Prusty, Roll No. 413LS2048, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I do hereby declare that the Project Work entitled **"APOPTOSIS AND AUTOPHAGY INDUCTION IN T98G GLIOBLASTOMA CELLS WITH CONCANAVALIN A."**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Sujit Kumar Bhutia, Head of Department, Department of Life Science, National Institute of Technology, Rourkela, Odisha. I have done this project for the partial fulfilment of requirement of my course curriculum of Master of Science, in Department of Life Science, NIT Rourkela.

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LIST OF ABRREBATIONS

%: Percentage

°C: Degree Celsius

ConA: Concanavalin

PCD: Programmed cell death

PBS: Phosphate Buffer Saline

MTT: 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]

DAPI: 4', 6-diamidino-2-phenylindole

FITC: Fluroescien Isothiocyanate

PARP: Poly (ADP-ribose) polymerase

AO: Acridine orange

GFP: Green fluorescent protein

OD: Optical Density

DMEM: Dulbecco's Modified Eagle Medium

MEM: Minimum Essential Medium eagle

DMSO: Dimethyl Sulfoxide

FBS: Fetal Bovine Serum

BSA: Bovine Sarine Albumin

gm.: Gram

Hrs: Hours

Kg: Kilogram

L: Liter

M: Molar

mg: Milligram

Min: Minute

mL: Milli Liter

mM: Milli molar

µg: Micro gram

ABSTRACT

Concanavalin A (Con A) is a lectin with mannose/glucose binding specificity depicts variety of biological science functions such as, study of hormones/receptor, mitogenic T-cell activation, characterizing normal and malignant cells as well as positive or negative staining bacteria, as lectin affinity chromatography, as an immobilization matrix, etc. The present study evaluated the potential role of Concanavalin A, anti proliferative effect in glioblastoma cell line i.e. T98G cells. The lectin Con A was isolated by salting-in and salting-out procedure and purified by affinity chromatography using sepharose 4B column. The protein was estimated and lyophilized for the in-vitro study of Con A. Initially, the cell viability assay (MTT assay) was done in a normal and the cancer cell for in-vitro study, IC-50 value was determined. Based on this, apoptosis and autophagy were studied using DAPI staining, Annexin V FITC staining, western blotting, Caspase 3/7 Glo assay, Acridine orange staining and GFP-LC3 transfection. In conclusion, we demonstrated that Con A induces both apoptosis and autophagy in a dose dependent manner in glioblastoma. Review of literature shows the molecular mechanism in hepatoma and Hela cells, but it is unexplored in T98G cell. Hence as the novelty of work we tried to explore the anti-proliferative activity of Con A in glioblastoma cell. Its molecular mechanism can be further studied for clinical treatment of cancer.

Key words: Lectin, Con A, Glioblastoma (T98G), Apoptosis, Autophagy

CHAPTER 1

INTRODUCTION

1. INTRODUCTION:

Lectins are ubiquitous carbohydrate-binding protein, which are non-immunoglobulin in origin and have one non-catalytic domain that reversibly binds to specific carbohydrates without altering their structure. Lectins are profoundly found in plants. Legume lectins are well known among them. Because of its carbohydrate binding property it has variety of functions, such as, cell adhesion (Lis and Sharon, 1989), mitogenicity (Moreira et al., 1991), different cell identification and differentiation, anti-proliferative activity, etc. Concanavalin A (Con A) is protein, or legume lectin, isolated from legume Jack bean or *Canavalia ensiformis*, of molecular weight 104kDa (Sumner and Howell, 1936). Con A binds specifically to the non-reducing terminal α-D-mannosyl and α-D-glucosyl groups of sugars, glycoproteins, and glycolipids. It is a homotetramer, with 237 glycated amino acids in each subunit. It has a saccharide binding site and has two metal binding sites i.e. with Ca^{+2} or Mn^{+2} (Hardman and Ainsworth, 1972).

Con A has a variety of functions for its specific carbohydrate binding property, such as, blood typing, bacteria identification by binding specifically to liposaccharides, mitogenicity that activates T-cells, immobilization matrix, cell adhesion, removing contaminants, hormone receptor studies, characterization of certain normal and malignant cells, cell division, anti-immflamatory, toxicity that damage intestinal mucosa, etc. This toxicity effect triggers the programmed cell death (PCD) (Liu et al., 2010; Dwyer and Johnson, 1981; Edelman, 1975; Picken and Beachman, 1975). Programmed Cell Death can be of two types- 1. Type I is Apoptosis (self-killing), which is characterized by morphological changes, for example- nuclear fragmentation, condensation of chromatin, membrane blebbing and apoptotic body development. Caspase-dependent apoptosis process has two pathways, i.e. extrinsic and intrinsic. Intrinsic pathway, the mitochondrial innervated pathway where as extrinsic pathway is a receptor mediated pathway (Jin and El-Deiry, 2005). 2. Type II is Autophagy (self-eating), which removes unwanted components of the cell by lysosomal process (Johansen and Lamark, 2011).

Con A shows a remarkable antiproliferative activity as well as, it induces apoptosis in fibroblast cells, i.e. balb/c 3T3 and human gingival fibroblasts (HGF) (Kulkarni and McCulloch, 1995) and report also shows, apoptosis is induced in A375 cells. The pathway is a caspase-dependent and also mitochondrial apoptotic pathway, in which mitochondrial transmembrane potential

subside, cytochrome *c* comes out and caspase is activated (Liu et al., 2010). Con A is inhibitory or cytotoxic to hepatoma cells, which is by autophagy. As, Con A binds to the glycoproteins of hepatoma cells and internalized to mitochondria by signaling pathway, which indicates this autophagy is a mitochondrial autophagic pathway (Chang et al., 2007). Thus in this report, we isolated and purified the protein Con A and then the effect of Con A treatment with different concentration was checked on the glioblastoma cell line T98G and the induction of apoptosis and autophagy was examined.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE:

The proteins in nature with activity to bind specific sugar moiety, to agglutinate erythrocytes are eventually named as **Lectins.** "Lectin" is derived from the Latin word "*Legere"*, that means "to select" as we know lectins are very specific to the site for binding. The proficiency of reorganization and binding of specific carbohydrate structures is the biological function of lectins (*Goldstein et al., 1986*). Goldstein described lectin as "a carbohydrate-binding protein or glycoprotein of non-immune origin which agglut
inates or precipitates glycoconjugates or both". These are carbohydrate-binding proteins which bind to glycoproteins, glycolipids, and also polysaccharides (Goldstein and Hayes, 1978) which mediates various kind of biological processes by binding to different sugar moiety (Lis & Sharon, 1998; Vijayan & Chandra, 1999). Lectins are the glycoproteins that not only specifically bind to monosaccharides by distinguishing different monosaccharides but also bind to specific oligosaccharides (Peumans and Damme, 1995) by detecting significant difference in complex carbohydrate structure.

Initially lectins were isolated from plant seeds; these were known to be ubiquitously distributed in nature (Sharon and Lis, 1989). Lectins are sufficiently found in plant parts such as vegetables, roots, barks, bulbs, rhizomes, tubers, fruits, seeds, etc. but in case of nuts, grains, seeds and beans contains highest amount (Lis and Sharon, 1986; Adenik et al., 2009; Wong et al., 2008; Audrey and Sharon., 2002). Researcher studied the 3-D structure and isolated lectins from different origins like plants, animals, fungi, lichens, bacteria (Liener, 1976; Hapner and Robins, 1979; Damjanov, 1987; Sharon and Lis, 1989). Out of these plant lectin are well studied and contribute significantly to biological research because –

1) These are readily accessible carbohydrate binding proteins and,
2) Easy for the examination, separation and purification.

2.1. Classification of lectins:

Because of their varying carbohydrate-tying specificities, lectins are classified into distinctive sorts in light of diverse standards. Contingent on the general structures of the full grown plant lectins, they can be doled out into the accompanying subdivisions (Damme et al., 1998):

1. Merolectins [monovalent with the single – sugar tying domain eg- Hevein (Van Parijs et al., 1991)]
2. Hololectins [two homologous mannose/glucose binding domains that can bind to same or or basically comparable sugars]
3. Chimerolectins [fusion proteins with multi sugar – binding domains]
4. Superlectins [at least two carbohydrate binding areas that perceive basically random sugars]

Contingent on the atomic structure, lectins are subdivided into 3 distinct sorts:

- Simple lectins, [few subunits of sub-atomic weight 40 kDa or less with extra tying areas eg: Galectins (S-lectins)]

- Multi-area lectins, [composite molecules with an extensive variety of molecular weights, having a few sorts of domains]

- Macromolecular get-togethers. [Found in microbes as pilli, these are filamentous, heteropolymeric organelles comprising of distinctive sorts of sub-units masterminded helically (Ofek and Doyle, 1994)]

]

Besides, plant lectins have also been classified according to their sugar-binding specificities into:

a) polyspecific (interfer with more than one sugar) or
b) monospecific (interfer with a single sugar) (Vijayan et al., 1999; Barre et al., 2001).

Investigation of accessible arrangements has prompted their grouping into seven groups of evolutionary related proteins. A few lectins are yet to be arranged into gatherings on the grounds that they don't fit into any of the present characterization frameworks and some grouping data is unavailable.

2.2. Plant lectins:

Leguminosae is the best-described group of plant lectins (Toms, 1971). Different groups of plant lectins are the Gramineae , Solanaceae, Euphorbiaceae and so on. These lectins vary extensively as far as essential/optional/tertiary structure. Lectins in plants are available fundamentally in seeds, cotyledons and parts (Van Damme et al., 1998). Plant lectins have viable biological activity and happen in nourishments like wheat, corn, tomato, banana nut, kidney bean, soybean and numerous more. In light of the amino corrosive groupings of protein, plant lectins are sub-partitioned into 3 unique sorts. These include:

a) Legume lectins - They comprise the largest family belonging to the family-Leguminosae. The legume seed/beans lectins are present in the storage protein vacuoles (Etzler, 1986). Occur in dimeric or trimeric forms, each subunit (25-30 kDa) having one carbohydrate binding sites. e.g., Concanavalin A (first lectin to be purified and isolated) (Sumner and Howell, 1936) and Phytohemagglutin.[figure-1]

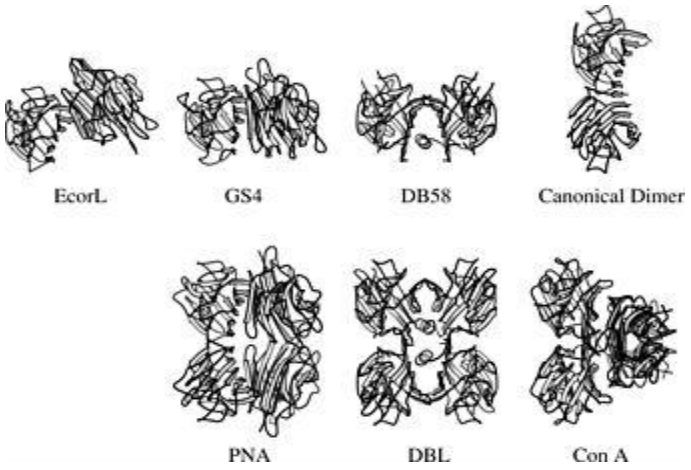

Figure-1: Quaternary structures of some legume lectins. Considering, one subunit in the same orientation for all structures to compare (wikipedia by Tomixdf).

b) Monocot mannose-binding lectins – These are mannose-specific lectins and are present in monocot families like Alliaceae, Araceae, Bromeliaceae, Liliaceae, and Orchidaceae (Van Damme *et al*, 1998). For example- *Galanthus nivalis* agglutinin (GNA), the first lectin to be crystallized and analyzed [figure-2].

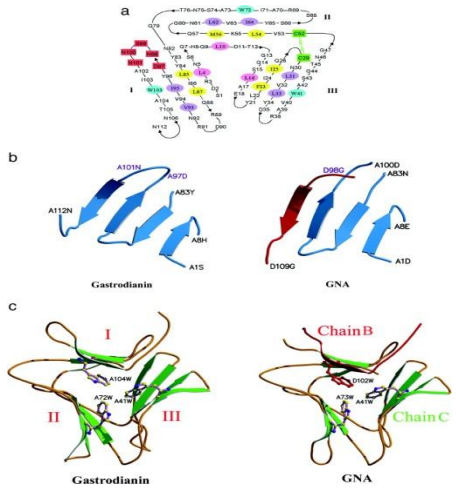

Figure-2: Structural organization of subdomain I in monomeric gastrodianin through the C-terminal self-assembly in comparison with that of GNA via the C-terminal exchange. (Liu W et al., 2005)

c) Chitin-binding lectins – Chitin specific binding lectins are found in plant families like Gramineae, Solanaceae, Phytolaccaceae, Urticacea, Viscaceae and Papavaracaceae (Peumans *et al*, 1996; Raikhel *et al*, 1993). These proteins consist of one hevein domain [figure-3].

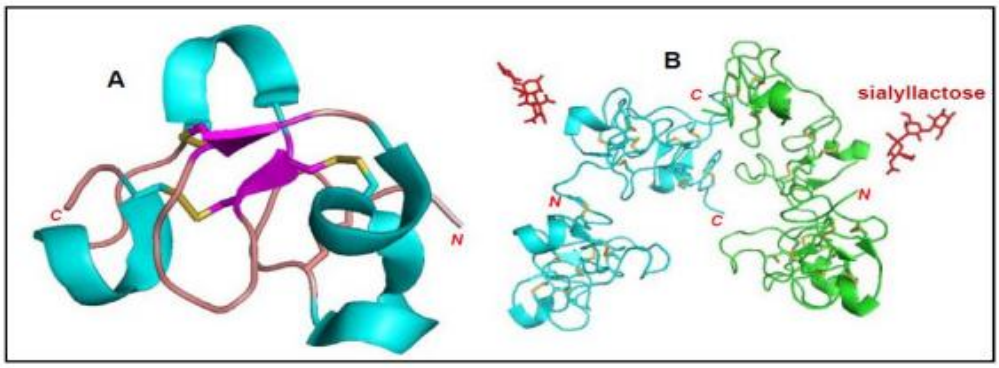

Figure-3**: A.** Three dimensional structure of hevein (PDB code 1Q9B). α-helices are colored in cyan and β-sheets are colored in magenta. The disulfide bonds are shown in yellow. **B.** WGA complexed with sialyl lactose (PDB code 1WGC).

2.3. Functions of lectins:

Lectins have commonly been used as molecular tools in several disciplines of biology and medicine. Lectins have the ability to distinguish carbohydrate determinants in human blood cells, so blood typing can be performed by lectins. Lectin associated haemagglutination assay and the ability of different oligosaccharides to act as inhibitors was determined by the microtitre V plates by using rabbit erythrocytes (Lechaire & Barondes, 1978). Lectins are used in immunological studies as a tool because at low concentrations, some lectins are mitogenic to peripheral blood lymphocytes. (Moreira et al., 1991). Used to activate lymphocytes and induces proteins like enzymes, interleukins or cytokines (Kilpatrick, 1991). Lectins analyze the carbohydrates and differentiate between the gram-positive and gram-negative bacteria. Bacteria contains the cell wall composed of peptidoglycan, teichoic acids (Gram-positive organisms), and Lipolysaccharides (Gram-negativeorganisms) (Ito et al., 1999). Lectin decreases the telomerase activity and suppresses angiogenesis. It modifies the cell cycle by inducing non-apoptotic G1-phase accumulation mechanisms. Plant lectins have also involvement in cell separation and bone marrow transplantation (Reisner et al., 1978, 1983). It has been used as carriers in drug delivery such as delivery of chemotherapeutic agents. Consumption of lectins alters normal growth inanimals including human. It has been reported that the lectins influences the nutrient intake (Liener, 1986).Lectin is very poisonous in nature. It cannot be degraded by digestive enzymes, so intake of food that contains lectin causes many health disorders like vomiting, diarrhea etc (Vasconcelos *et at*., 2004). However, some lectins, such as ConA and Ricin are extremely cytotoxic to cells at higher concentrations and may be used for cancer treatment.

CHAPTER 3

INTRODUCTION TO CONCANAVALIN A

3. INTRODUCTION TO CONCANAVALIN A (ConA):

Concanavalin A (conA) is a carbohydrate-binding protien belonging to the group of protein called lectin. It is a legume lectin, as purified and crystallized originally from the seed of a legume Jack bean or *Canavalia ensiformis* a species from Diocleinae subtribe. Con A is the first crystallized by Sumner and Howell (1936), it was to be commercialized. As a carbohydrate-binding protien, it binds to the nonreducing terminal α-D-mannosyl and α-D-glucosyl groups of sugars, glycoproteins, and glycolipids (Goldstein and Poretz, 1986). Con A have a 3-D structure (Becker *et al*., 1975) with homodimer or homotetramer subunit (Loris et al, 1998) each consisting of 237 glycated amino acids (Olson and Liener 1967) that has one saccharide binding site and two metal binding sites i.e., transition metal (Kalb and Levitzki, 1968) like Ca^{+2} or Mn^{+2} (Hardman and Ainsworth, 1972) [figure-4].

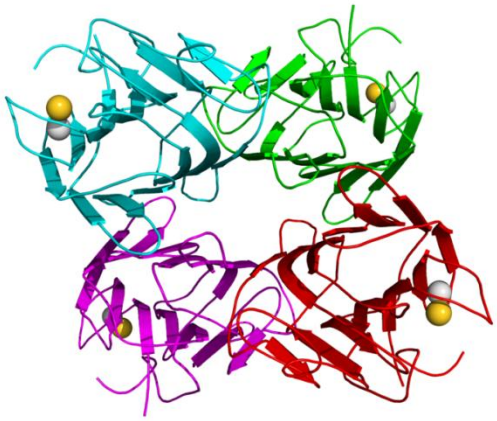

Figure-4: Crystallographic structure of concanavalin A, showing homotetramer subunits. Graphic rendered with PyMol.

3.1. Various roles of conA:

Because of conA specific binding to carbohydrates, it has various roles:

- It agglutinates the red blood cells by binding to immunoglobulin glycopeptides (Kornfeld and Ferris 1975).
- It specifically binds to the carcinoembryonic antigents and defends (Brattain *et al*. 1975).
- Con A has mitogenic activity, which activates lymphocytes T-cell (Powell and Leon, 1970).

- With its specificity it binds to liposaccharides of bacteria (Picken and Beachman, 1975).
- Con A induces oocyte maturation-inducing substance in starfish follicle cells (Kubota and Kanatani, 1975).
- In the processes such as immobilization and isolation (Anderson and Lee-Own, 1974) and removing contaminants (Edelman, 1975) conA can be used.
- Useful for studying cell division, cell surface receptors, immune regulation by various immune cells (Dwyer and Johnson, 1981).
- Con A has anti-cancer activity because of its specific carbohydrate binding nature, as it cannot proliferate to form tumor (Liu et al., 2010).
- Con A can inhibits liver nodule formation by oral injection, this indicates the chemopreventive nature of Con A for cancer treatment (Rosenberg et al., 2004).
- Con A is toxic in nature as they damage intestinal mucosa and can trigger both autophagy and apoptosis in cancer cells (Liener, 1995). Con A has a high agglutination activity towards cancerous cells or transformed cells due to the presence of their exposed binding sites, as compared to normal cells (Inbar and Sachs, 1969).

3.2. Apoptosis:

Cells have an intrinsic mechanism to kill themselves, one of them is the apoptotic cell death , which is a form of programmed cell death to describe a morphologically different form of cell death, 21luorescent21o by several morphological features such as chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and apoptotic body formation. Apoptosis is mediated by intracellular cysteine proteases called caspases which share the ability to cleave their substrates after aspartate residues (Li and Yuan, 2008). There are two main pathways in apoptosis, extrinsic and intrinsic. Extrinsic pathway is a receptor mediated pathway where as intrinsic pathway is a mitochondrial mediated pathway [figure-5] Both pathways eventually leads to the activation of proteolytic cascade. Apoptosis is also involved in the regulation of many pathological processes including cancer. One of the hallmarks of human cancers is the evasion of apoptosis promoting tumor formation and progression (Jin and El-Deiry, 2005).

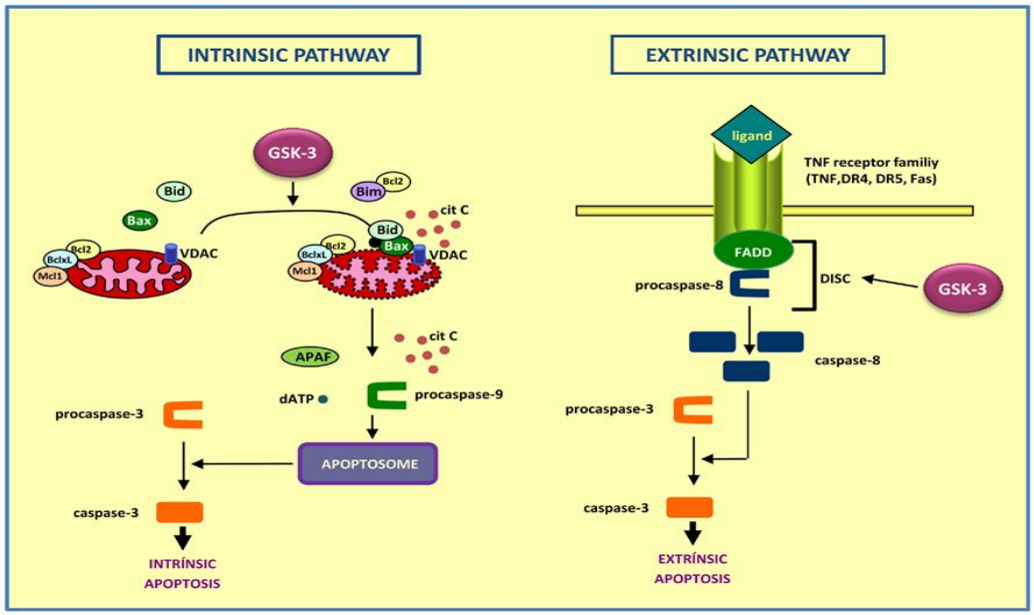

Figure-5: Diagram showing the intrinsic (left) and extrinsic (right) apoptotic pathways**. (**Gomez-Sintes et al, 2011)

3.4. Con A induced apoptosis and related molecular mechanisms:

Con A, has been drawing a great interest to its anti-proliferative activity. It was reported that Con A induces apoptotic programmed cell death (Chen, M et al., 2002), because of carbohydrate-binding property. It has been reported that con A induces apoptosis in human melanoma A375 cells in a caspase-dependent manner by mitochondrial pathway (Liu et al., 2009), in balb/c 3T3 (3T3) and diploid human gingival fibroblasts (HGF) (Kulkarni and McCulloch, 1995). It is also been reparted that Con A is associated with mitochondrial transmembrane collapse, cytochrome *c* release and caspase activation, which 22luorescen that con A induces self killing by mitochondrial apoptotic pathway (Liu et al., 2010). Con A treatment to neurons shown some morphological changes in cell such as chromatin 22luorescent22, nuclear fragmentation and membrane blebbing. These are characteristics features of apoptotic cell death (Cribbs, D.H. et al., 1996). P53 is a tumor 22luorescen gene known to regulate cell cyle and apoptosis, this gene is mutated in cancer cells. Normal cell on treatment with con A, delayes the G2/M phase then by 22luoresce G1 phase. So the cells lack p53 and undergo apoptosis (Amin et al., 2007). Thus from the above distinctive results of treatment of different tumor cells and normal cells with con A, induces apoptosis that too by distinct molecular mechanism (caspase mediated mitochondrial

pathway), suggests that Con A is a novel molecule to induces apoptosis and thus treat cancer by affecting tumor cell growth.

3.5. Autophagy:

Autophagy (auto- 'self'and phagy – 'eating'), the self eating catabolic process, type-II 23luorescen cell death, which degrades unnecessary own cell components by lysosomal process.. Autophagy is a fundamental regulatory homeostatic process in both normal and stressful situation, which has a significant role in cell survival mechanism (Verschuer et al., 2012). Autophagy mainly removes the unwanted cytoplasmic 23luorescen and recycles the cellular components, such as organelles turnover. It protects cells during nutrient starvation.

Mechanism of autophagy is a dynamic pathway regulated by several genes. It is a nonselective degradation process which directly engulfs the cytoplasmic organelles (Johansen and Lamark 2011). The complete process of autophagy is divided into three important steps i.e. nucleation, elongation and maturation [figure-6]. The stressed cells initiate the formation of phagophores, a double layer membrane. By molecular signaling process of autophagosome formation completes and maturation occurs when fuses with 23luoresce to form autophago-lysosome or autolysosome which degrades the cellular components.

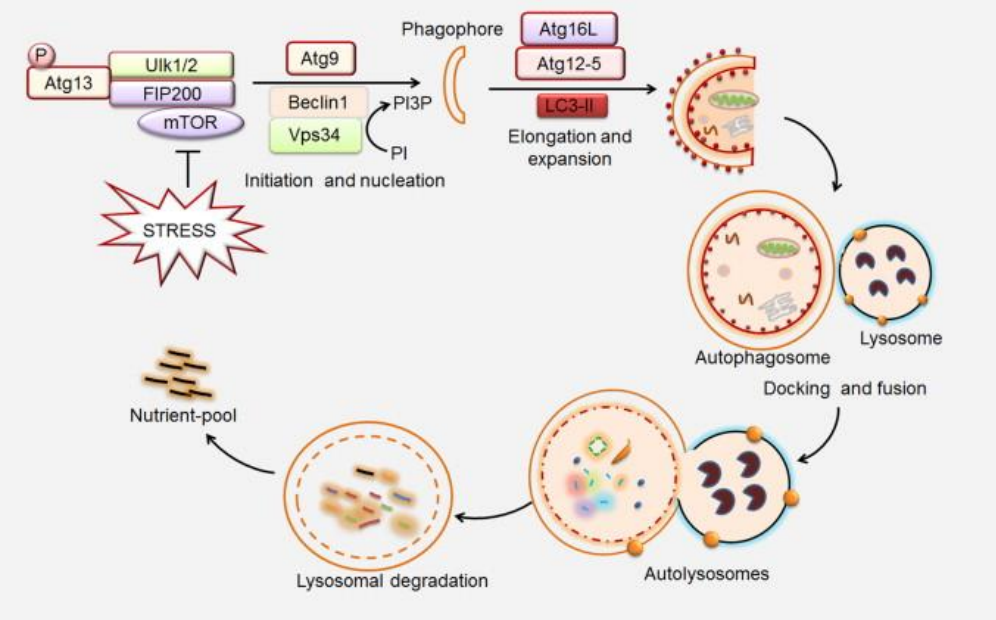

Figure- 6: The process of autophagy and its steps. (Panda et al., 2013)

3.6. Con A induced Autophagy and related molecular mechanisms:

Con A has an important influence on its anti-proliferative activity, because of specific sugar binding property, this helps to induce cell death. Con A was prefferntially internalized to the mitochondria via clathrin-mediated endocytosis (Lei et al., 2009), after binding to mannose moiety in the hepatoma cells. Thus con A induces autophagic programmed cell death by a mitochondria-mediated pathway (Chang et al., 2007). It was shown that Con A treatment with CT-26, ML-1 cell line various autophagic pathway noted, such as, formation of LC3-II, vesicles with double-layer and induction of BNIP3. In case of ML-1 cells it observed that autolysosome formation was takes place which indicates that Con A induced autophagy (Lei et al., 2007). Con A-like substances, particularly natural substances like edible vegetable plants, seeds have both immunomodulating and autophagy-inducing activities.

CHAPTER 4

OBJECTIVES

4. OBJECTIVES :

1) Isolation and purification of con A from the seeds Jack bean (*Canavalia ensiformis*).

2) Estimation of protein concentration by Lowry's method.

3) To check cell viavility and determine IC-50 value, by MTT assay.

4) To study apoptotic cell death induced by con A in T98G (human glioblastoma multiforma tumor):

1. DAPI staining
2. Annexin V FITC staining
3. Western blot –PARP assa
4. Caspase-3/7 Glo Assay

5) To study conA induce autophagy in T98G cells by:

- Acridine orange staining
- GFP-LC3 transfection

CHAPTER 5

RESEARCH METHODOLY AND MATERIALS

5. RESEARCH METHODOLOGY AND MATERIALS :

5.1. Materials:

5.1.1. Sample collection:

The dry *Canavalia ensiformis* (jack-bean) seeds were collected from the local area of Koraput, Odisha, India.

5.1.2. Cell line :

The whole experiment was carried out by using the human glioblastoma multiforma tumor cell line T98G, which was collected from National Center for Cell Science (NCCS), Pune. The cells were cultured in Modified Eagle Medium (MEM) and supplemented with antibiotic-antimycotic and 10% fetal bovine serum.

5.1.3. Chemicals :

Sodium carbonate (Na2CO3), Sodium hydroxide (NaOH), Cupper sulphate (CuSO4), Glycine, Potassium sodium tartarate (KnaC4H4O6) were purchased from SRL, Sisco research laboratories Pvt. Ltd., Mumbai. Sodium chloride (NaCl) was purchased from Fischer scientific. Ammonium per sulphate (APS), Acrylamide, bisacrylamide, Sodium dodecyl sulphate (SDS), ethanol, Bovine serum albumin (BSA), N, N, N', N'-tetramethylenediamine (TEMED), potassium sodium tartarate (KnaC4H4O6) and Tris were purchased from Sigma Aldrich, USA. . Bovine serum albumin (BSA), β metacarpoethanol was purchased from Himedia, Mumbai, India. Potassium hydrogen phosphates (K2HPO4), Potassium Dihydrogen Phosphate (KH2PO4), were purchased from S.D. fine 28luor. Ltd., Mumbai. Ethanol was from Trimurty Chemicals, India. Acetic acid and agarose were purchased from Himedia, Mumbai. Cell culture media viz. MEM, DMEM were purched from the Gibco. Silver nitrates, Sodium thiosulphate, Methanol, were purchased from nice chemicals Pvt .Ltd. India. Pre stained molecular weight marker were purchased from Bio-Rad, India. Trypsin & antibiotics were purchased from the Hi-media. Lipofectamine 2000® was purchased from Invitrogen, USA. Acridine Orange, DAPI, MTT and Annexin V-FITC were purchased from the Sigma.

PARP from Cell Signalling Technology Pvt Ltd. GFP-LC3 from Addgene 29luorescent29. 96 well microplate, T25 and T75 cell culture plates were purchased from tarson, India.

5.2. Methods :

5.2.1. Isolation and purification of con A from jack bean seeds:

Extraction of con A was done from seeds of jack bean. 53g of jack bean seeds were taken, and soaked in 130ml of PBS solution overnight. The next day, it was grinded to make a paste. The total weight of paste was found to be 190.53g. Then they were centrifuged at 7500 rpm for 20 minutes at 4°C. The supernatant obtained from homogenous paste was crude (2ml were kept). Then 30% cut-off was made in 90ml of supernatant by saturating them with 15.84g of ammonium sulphate and stored at 4°C for 12 hours. Then they were centrifuged at 7500 rpm at 4°C for 20 minutes and 2ml was kept. Pellet was discarded and 90% cut-off was made with 78ml of the supernatant by saturating them with 34.164g of ammonium sulphate and stored at 4°C overnight. The next day it was centrifuged at 7500 rpm at 4°C for 20 minutes. The pellet was mixed with 25ml distilled water and vortexed and made upto 50ml. These samples were kept for dialysis in distilled water for 2 days and in PBS for 1 day. Then 45ml of the sample centrifuged at 7500 rpm at 4°C for 20 minutes and 50ml of supernatant was collected and stored at 4°C, after syring filteration.

5.2.2. Affinity Chromatography:

Affinity chromatography technique is used for the purification of a molecule of interest from complex mixtures. It is based on highly specific reversible biological reactions between two molecules such as enzyme and substrate, receptor and ligand, or antibody and antigen. Affinity chromatography technique using carbohydrate ligands or matrices for the purification of lectins (or glycan-binding proteins) is known as carbohydrate affinity chromatography. The maltamyl sepharose bead column was used for lectin (con A) binding. The filtered and stored dialyzed sample was used for affinity chromatography by Akta prime [figure-7]. Initially, for cleaning the Akta, it was washed with PBS and 20% ethanol. Then the maltamyl sepharose beads [figure-8] were washed in PBS solution, its O.D. was measured at 280 nm, with UV light exposure. Then 45ml of protein sample was loaded on the maltamyl sepharose beads and

O.D. of the flow through was measured at 280 nm. Then the maltamyl sepahrose beads were again washed with PBS solution to remove the undesired proteins. 5ml of 0.6M maltose solution was loaded on the Maltamyl Sepharose 4B beads and O.D. of the eluent was measured at 280 nm. Since the maltose sugar are bounded to eluent sample, so they can be removed by dialysis in 2 liters of PBS solution and stored at 4°C for 1 day. Then the sample was stored at 4˚C for lyophilisation.

Figure-7 Akta prime- affinity chromatography Figure-8 Maltamyl Sepharose 4B bead

5.2.3. Estimation of protein by Lowry's method:

Principle:

The Lowry assay is based on the reaction of cupric or copper [II] ions with peptide nitrogen[s] under alkaline conditions (the Biuret test). Protein samples are mixed with an alkaline solution containing copper sulphate (Cu2+ ions) which reacts with peptide bonds to produce monovalent Cu+ ions – strong reducing agents. The monovalent copper ions and the aromatic amino acids (tyrosine, tryptophan, and cysteine) reduce the Folin-Ciocalteau phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue. The blue color of the dye can then be measured at an absorbance of 600 nm. The color produced is a direct reflection of protein concentration and, with the use of standards, can facilitate determination of protein concentration.

Protocol:

The concentration of crude, 30% cut-off, 90% cut-off and 90% cut-off affinity was estimated by Lowry's method. Bovine serum albumin (BSA) was used as the standard protein.

The reagents required:

1. BSA stock solution (1 mg/ml)
2. Analytical Reagents

Reagent X: Sodium hydroxide (0.5%)

Sodium carbonate (2%)

Reagent Y_1: 1% copper sulphate

Reagent Y_2: 2% sodium potassium tartarate

The analytical reagents were prepared by mixing 1ml of both reagents Y_1 and Y_2 and 100 ml of reagent X.

3. Folinciocalteau reagent: 1N of this reagent was prepared by mixing equal volume of the reagent and water i.e., 5 ml of the reagent + 5 ml of distilled water.

Different concentration of BSA solutions were taken from stock solution and distilled water was added to it and made up to 200µl. Then 2ml of Lowry's reagent was added and incubated for 15 minutes. Then 200µl of Folin reagent was added and incubated for 30 minutes. Their O.D. was taken at 595nm. The concentration of the unknown protein of crude, 30% cutoff, 90% cutoff and affinity sample was plotted in a graph taking absorbance in Y-axis and concentration in X-axis.

5.2.4. MTT assay (to assess the effect of Con A on T98G cells):

Principle:

MTT [3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay is a colorimetric assay to check cell survivability, which measures the enzymatic reduction of yellow colored tetrazolium dye (MTT) to purple colored formazan product by the mitochondrial enzyme succinate dehydrogenase. So, when the MTT penetrates the cell membrane and moves into the mitochondria, it gets reduced to an insoluble, dark purple colored formazan product, which occurs only in a live cell. When solubilised by DMSO (an organic solvent), it imparts purple colored with the solubilisation of formazan. Then the absorbance of the solution is measured spectrophotometrically at 500- 600 nm.

Protocol:

In this study, MTT assay was done to evaluate the cytotoxic effect of con A in varying concentration on T98G and HaCat cell line. The cells were subcultured in T25 culture flask and trypsinised when they attended 80-90% confluency. The cells were then seeded in 96 well plates. After 24 hrs, the cells were treated with varying concentration of con A with 6 replicates in each concentration and kept for 72 hrs incubation. MTT was then added to the wells and kept for about 4 hrs. Then, DMSO was added to dissolve the insoluble purple formazan and absorbance was taken at 562nm using Perkin Elmer 2030 reader.

5.2.5. DAPI staining (on treatment of Con A on T98G cells, by morphological changes of nucleus, for the study of apoptosis):

Principle:

DAPI or 4', 6-diamidino-2-phenylindole is a fluorescent stain, which binds strongly to A-T rich regions in DNA. Apoptotic cell show typical features, such as, reduction in cell-size, membrane blebbing, nuclear shrinkage, chromatin condensation and nuclear fragmentation. Chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be detected under fluorescence microscopy, after suitable staining of nuclei with DNA-specific fluorochromes such as DAPI. DAPI can go through an intact cell membrane therefore it can be preferred to stain both live and fixed cells. However, it has less efficiency in passing through membrane in live cells and therefore the potency of the stain is lower.

Protocol:

Previously seeded T98G cells were treated with different concentrations of Con A followed by incubation for 24 hrs. Then old media is dispensed out, the cells were treated with 10% paraformaldehyde for 20 mins and was removed. Then washing with PBST was done followed by washing with PBS twice. Next, DAPI (1X) stain was added to the cells and

incubated in dark for 5 mins. Then the cells were washed with PBS for 3times and observation was taken under Olympus 1X71 microscope.

5.2.6. Annexin – V FITC staining (to study apoptosis, through membrane phospholipid exposed):

Principle:

FITC Annexin V staining can discern apoptosis at an earlier stage. Morphological characters seen in case of apoptotic cell death are loss of plasma membrane asymmetry, chromatin condensation and nuclear shrinkage. The membrane phospholipid i.e. phosphatidylserine (PS) is transported from the inner to the outer leaflet of the plasma membrane of apoptotic cells. Thus, apoptosis exposes PS to the external cellular environment. Annexin V is a Ca2+ dependent phospholipid-binding protein with a molecular weight 35-36 kDa. Annexin V has a high affinity for PS, and binds the cells with revealing PS. Annexin V may be conjugated with Fluroescien Isothiocyanate (FITC) to detect PS on membrane surface. Annexin V with FITC causes membrane integrity loss and by which cell death occurs, due to apoptosis and necrosis. Hence, FITC Annexin V staining is usually used in combination with a vital dye like propidium iodide (PI). This process allows the researchers to spot easily the apoptotic cells and the results will be PI negative, FITC Annexin V positive for live cells.

Protocol:

T98G cells treated with different concentration of Con A was seeded and incubated for 24hrs in 12-well plates. Then the cells were washed with PBS for 2times and treated with 1X Annexin V binding buffer, followed by addition of Annexin V-FITC stain and incubated for 15 mins. Next, observed under fluorescence microscope. (Bhutia et al., 2010).

5.2.7. Western blotting:

Principle:

Poly (ADP-ribose) polymerase (PARP) is a protein of 113kDa molecular weight, involved primarily in DNA repair and programmed cell death (apoptosis). It is found in the cellular nucleus, and it is a well- known substrate of caspase-3, in the process of apoptosis. It plays a vital role as DNA-repair enzyme that transfers ADP-ribose units from NAD+ to different nuclear proteins including topoisomerases, histones, etc. Via poly ADP ribosylation, PARP-1 is accountable for management of cellular homeostasis, such as, cellular repair, cytoskeletal organization, transcription and replication of DNA and protein degradation. In response to DNA damage, PARP-1 activity is increased upon binding to DNA strand nicks and breaks. During apoptosis, PARP-1 is cleaved by caspase-3 between Asp214 and Gly215, resulting in the formation of an N-terminal 89 kDa fragment containing most of the DNA binding domain, which acts as an in-situ apoptotic marker. Hence, by giving antibody against this 89kDa fragment of PARP, we can study apoptosis.

Protocol:

Cell were seeded and treated with different concentration of Con A, incubated for 24hrs. These cells were collected by scrapping and lysis buffer was given to extract protein. The protein is estimated by Barford's method. Proteins were loaded on a 10% SDS-PAGE for isolation and then transferred to nitrocellulose or PVDF membrane. Incubated in blocking buffer (PBST with 1% BSA) for 2hours at RT, and then washed with PBST and primary antibody i.e. PARP1 polyclonal antibody (Pab) was added incubated at RT, at 4˚C overnight. After that, again washed with PBST and secondary antibody i.e. anti-human IgG conjugated with HRP at RT, at 4˚C for 2 hours. The membrane is washed with PBST and was developed with ECL.

5.2.8. Caspase 3/7 Glo assay:

Principle:

The Caspase- 3/7 Glo Assay is a homogeneous, luminescent, automated high through put assay which measures caspase-3 and -7 activities. These individuals from the cysteine aspartic acid- particular protease (caspase) family play vital role in apoptosis in mammalian cells. Caspase-3/7 substrate has the tetra peptide sequence (DEVD). Caspase activity present is directly proportional to the Luminescence. The Caspase- 3/7 Glo Reagent depend upon the properties of a thermo-stable luciferase enzyme, which is prepared to produce a stable "glow-type" luminescent signal and enhance performance in a wide range of assay conditions. The Caspase Glo-3/7 Assay generally shows caspase activity or apoptosis.

Protocol:

In this assay, 100µl of solution was taken in 1:1 ratio of caspase-3/7 Glo reagent volume to sample volume. The substances required for this assay are proteins, lysis buffer and caspase-3/7 reagent. Protein was estimated. After estimated 25µg protein was mixed with cell lysis buffer. After that, caspase-3/7 Glo reagent was mixed with the solution. After 6-8 hrs incubation in dark reading was taken in promega 20/20 luminometer.

5.2.9. Acridine orange staining (to study autophagy):

Principle:

Acridine orange (AO) is a nucleic acid staining fluorescent cationic dye. AO is useful for determining cell-cycle and it is permeable to intact cell membrane. It interacts with both DNA and RNA in the nucleus. It enters into lysosomes and other acidic compartments and becomes protonated. AO is an autophagic assay and is characterized by formation of AVOs (acidic vesicular organelles) viz. lysosomes and autophagolysosomes. After staining the cells with AO, AVOs can be estimated by flow cytometry. AO is a weak base that gets concentrated in

acidic spaces and produce red fluorescence. AVOs can be determined with increasing the intensity of red fluoresce which is proportional to the degree of acidity or 36luorescent36osomes formation..

Protocol:

T98G cells were seeded in a 12-well plate and were treated with different concentrations of Con A, incubated for 24 hours. After that, acridine orange (AO) was added at a final concentration of 0.5µg/ml for 15 min. Then observation was taken under the fluorescence microscope (Olympus IX 71).

5.2.10. GFP-LC3 transfection (to confirm autophagy, by analyzing the autophagosomes formation with LC3 marker protein):

Principle:

Molecular mechanism study of autophagy has led to several molecular marker proteins for autophagosomes. Marker proteins help us to trace the autophagic structure easily and accurately, just by imaging under fluorescent microscopy. LC3 (mammalian homolog of Atg8) is a widely used marker for autophagosome. To analyze autophagy GFP-LC3 is produced and studied.

LC3 is attached to both the inner and outer limiting membranes of autophagosomes, and the membrane interaction is mediated by a covalent bond conjugation to a lipid, phosphatidylethanolamine. A western blot shows two forms of LC3, LC3I and LC3II. LC3I is found in the soluble fraction and LC3II in the pelletable membrane fraction. Both LC3I and LC3II are seen in nonstarved cells, but during autophagy induction the proportion of LC3II increases.

Protocol:

T98G cells were seeded in 16mm petriplates to grow approximately 5×10^3. After 24hrs, cells were transfected with Pegfp-LC3(plasmid), using lipofectamine and incubated for 48hours. This lipofectamine creates pores in the membrane for the entry of plasmid. Then these cells were treated with different concentration of Con A i.e. 25µg, 50µg and 100µg. and incubated for 24hours and fluorescent imaging is done with 37luorescent microscope at 40X.

CHAPTER 6

RESULT AND DISCUSSION

6. RESULT AND DISCUSSION:

6.1. Affinity chromatography:

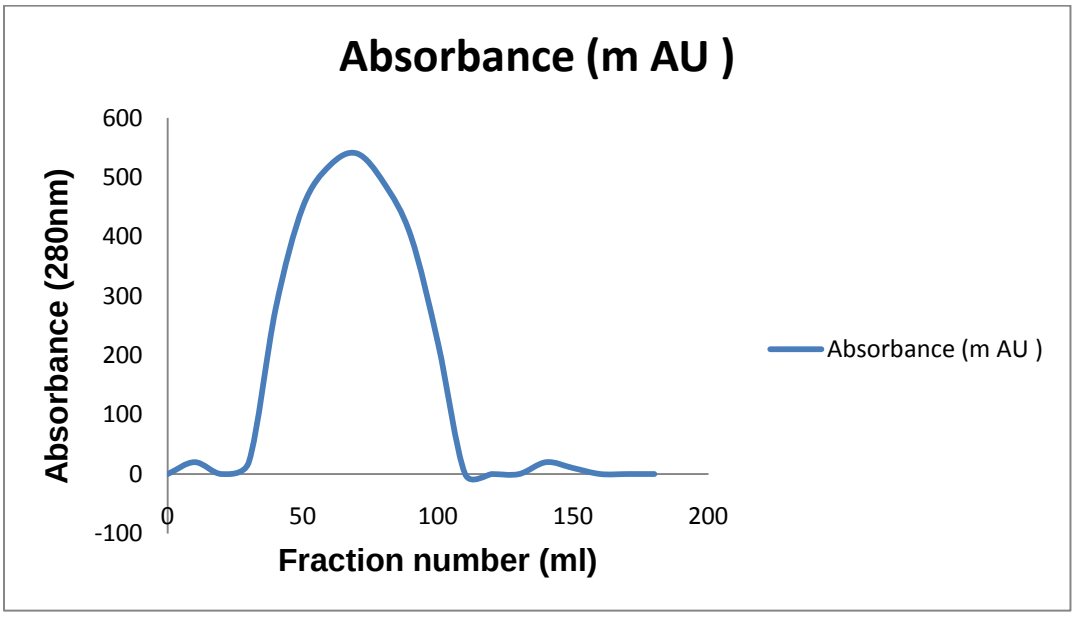

Figure-9: Affinity chromatography peak.

Inference:

The affinity chromatography peaks (Fig 9) obtained while isolating the Con A using maltamyl sepharose 4B column clearly indicates that after the elution of unbound proteins with PBS ,our lectin of interest i.e. a mannose-binding lectin (Con A) was eluted from the column by injecting 5ml of 0.6 M mannose.

6.2. Protein estimation by Lowry's method:

Estimation of protein concentration for crude, 30% cut off, 90% cut off and affinity fractions was carried out by Lowry's method and the following results were obtained (Table 1).

Table-1: Protein estimation by Lowry's method-

Samples	Volume (ml)	Concentration (mg/ml)	Total protein content (mg)
Crude	90	52.68	4741.2
30% cutoff	72	42.55	3063.6
90% cutoff	40	21.33	853.2
Affinity fractions	22	0.487	10.714

Inference:

By Lowry's method, the final protein concentration in the elute protein obtained after affinity chromatography was found out to be 10.714mg. The affinity purified fraction after dialysis was lyophilized and 10mg protein (approx.) in lyophilized powder, which was used for further characterization assays and tests for determining apoptotic and autophagic activity of the isolated Con A.

6.3. MTT cell survivability assay:

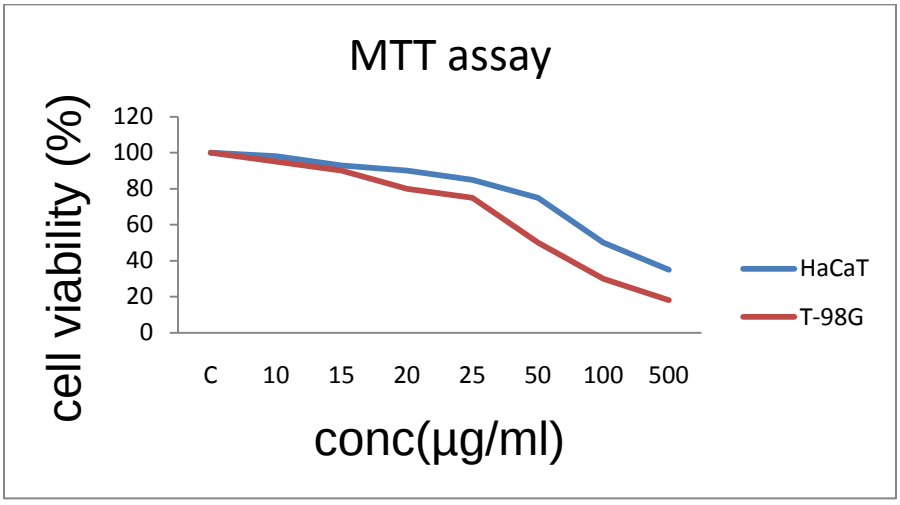

Figure-10: Graph showing cell viability of Con A treated to HaCaT and T98G cells.

Table:2 IC-50 values in µg/ml of different cell lines, after Con A treatment.

Cell line	IC-50 value (µg/ml)
HaCaT	**80- 100**
T-98G	**50**

Inference:

Con A was found to reduce the viability of T98G cell line at indicated concentrations with six replicates in each concentration (Figure-10).Moreover, the IC50 value for the normal human cell line i.e. HaCaT is significantly higher than that of cancer cell lines i.e. glioblastoma cells T-98G (Table-2). This indicates that Con A is selectively cytotoxic to cancer cell lines at lower concentrations and a significantly higher dose is required to inhibit the proliferation of normal human cells in vitro.

6.4. DAPI staining:

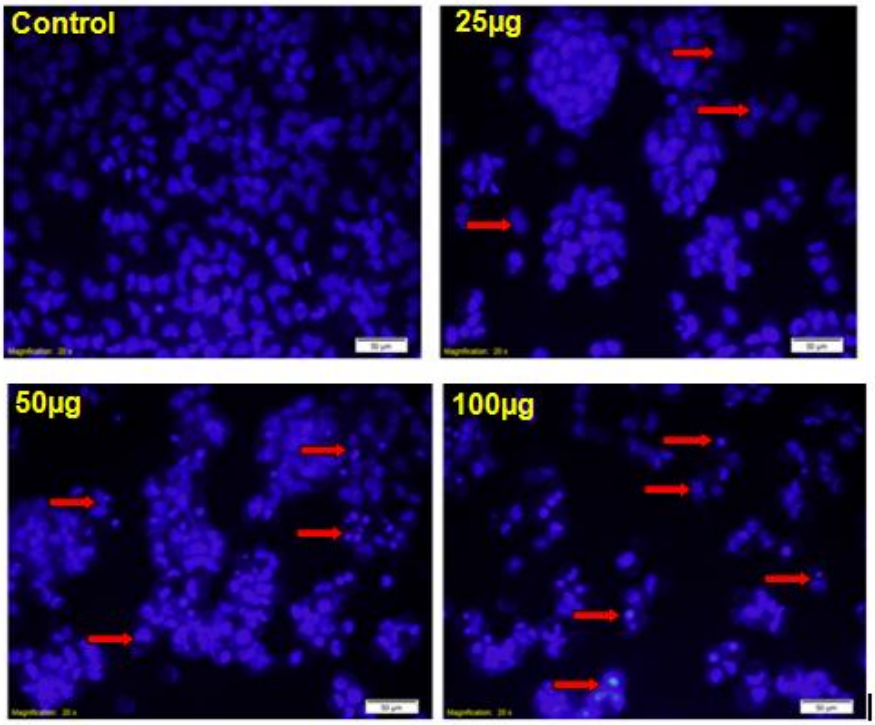

Figure-11 DAPI staining showing nuclear changes.

Inference:

With the increasing concentration of Con A in T98G cells the chromatin condensation and nuclear fragmentation also increases. At higher dose (100µg/ml) con A, more nuclear changes occur which depicting the apoptotic activity [Fig-11].

6.5. Annexin- V FITC:

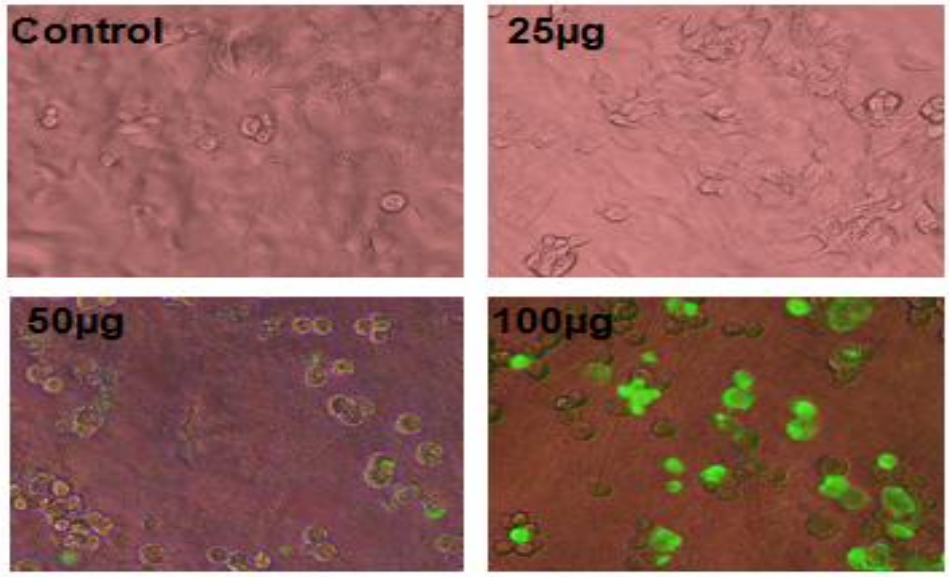

Figure-12 Annexin V- FITC staining images of T98G cells treated with Con A.

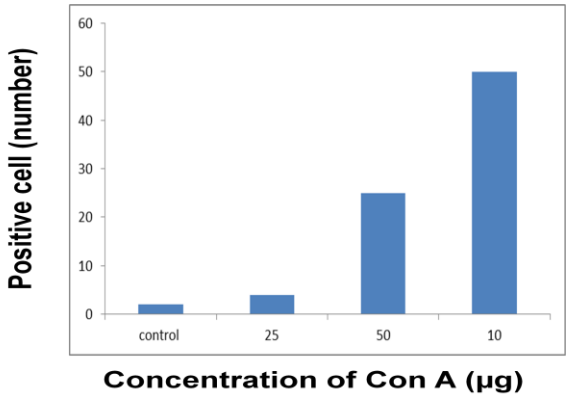

Figure-13 Graph of positive Annexin V FTIC showing cells with increasing concentration.

Inference:

Annexin V along with FITC (flurophore molecule) binds to the phosphotidyl serine (PS) present in the inner membrane and causes loss of membrane integrity exposing the PS, which signifies apoptosis. From these images it is observed that at higher concentration there is more membrane blebbing which shows more apoptosis is induced (Fig-12).. The graph plotted further shows a overall count of positive cell (fig- 13).

6.6. Western blotting to analyze PARP1:

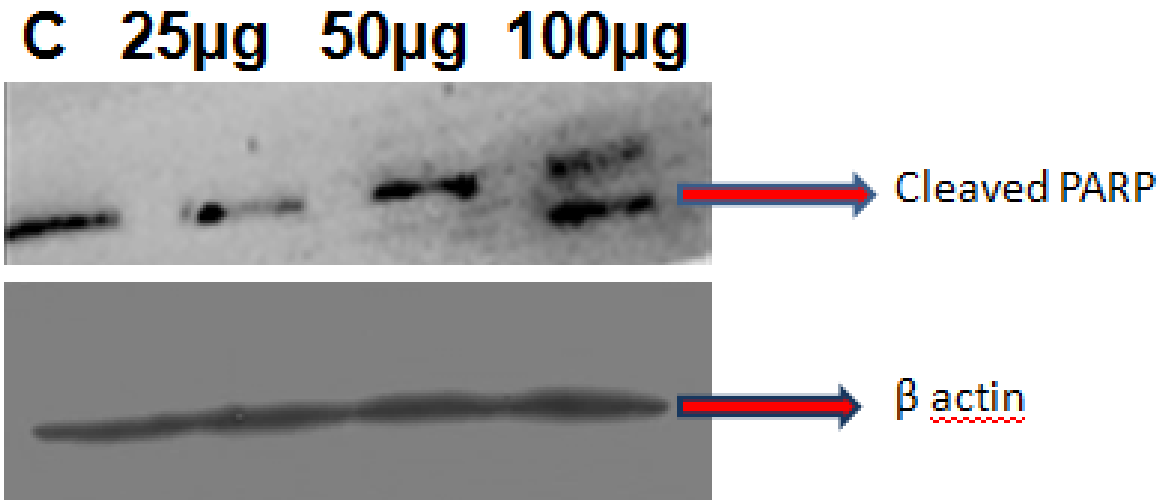

Figure-14 Western blot shows cleavage of PARP.

Inference:

As the dose of Con A increases, PARP is cleaved by the caspase activation to one 89kDa fragment. This shows [figure-14] that apoptosis is induced at higher concentration of Con A.

6.7. Caspase 3/7 Glo assay:

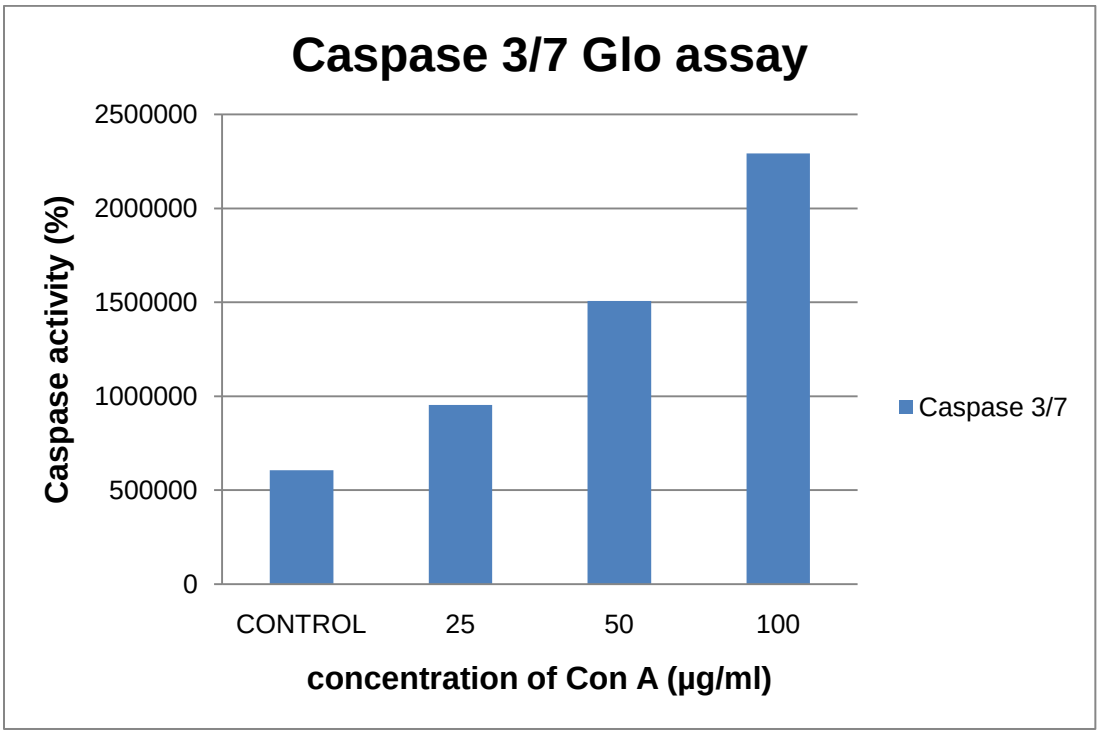

Figure-15: Graph showing caspase activity % with different concentration of Con A treatment

Inference:

Apoptosis is a caspase-dependent process, so the caspase-3/7 glo assay plays a major role in proving apoptosis induction. It was observed that the caspase activity increases accordingly with the increase in concentration of Con A. At higher concentration of con A i.e. 100µg/ml, more caspase activity is observed, which indicates more apoptosis. (fig- 15).

6.8. Acridine orange staining:

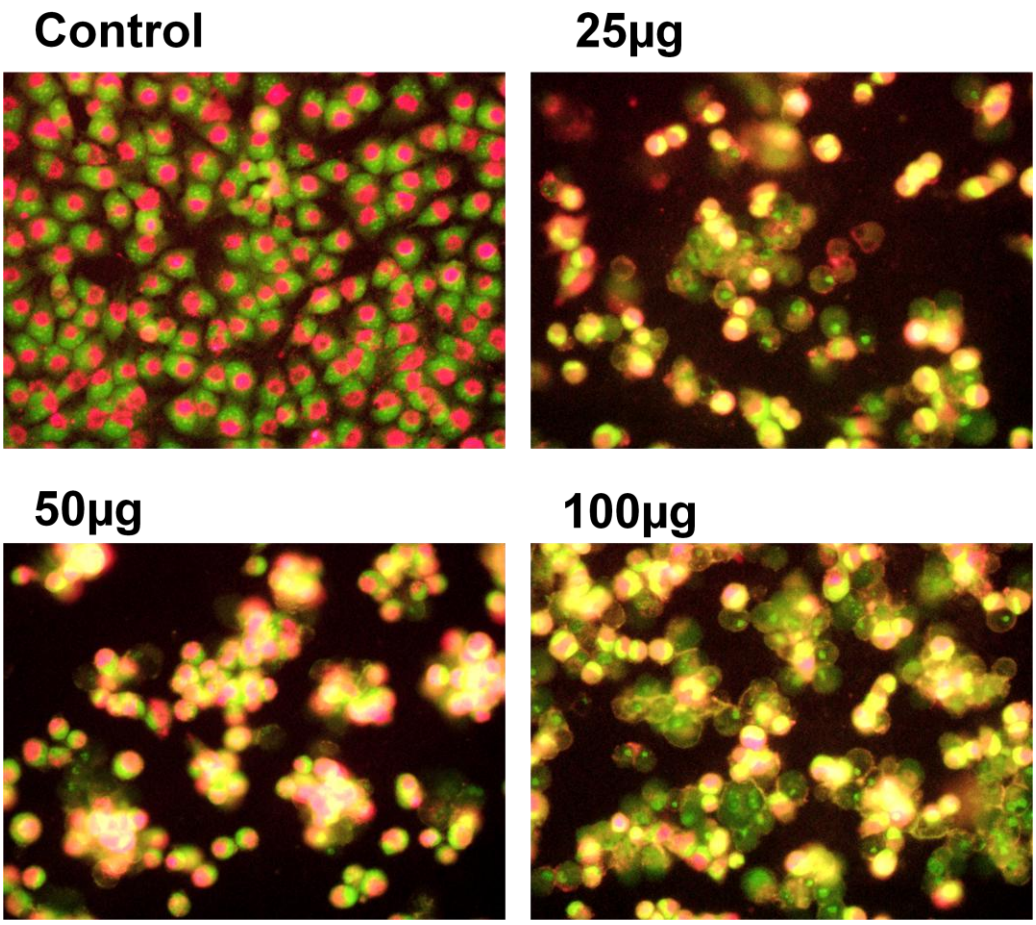

Figure-16: Images showing acridine orange staining of T98G cells with different Con A concentrations.

Inference:

Whencells are treated with different concentrations of con A in a dose dependent manner, the redness level increases accordingly. The shift of redness increase indicates the formation of autolysosome. So in higher dose i.e. in 100µg/ml there is more redness level which shows more autophagy in Hep-2 cells [Figure-16].

6.9. GFP-LC3 transfection:

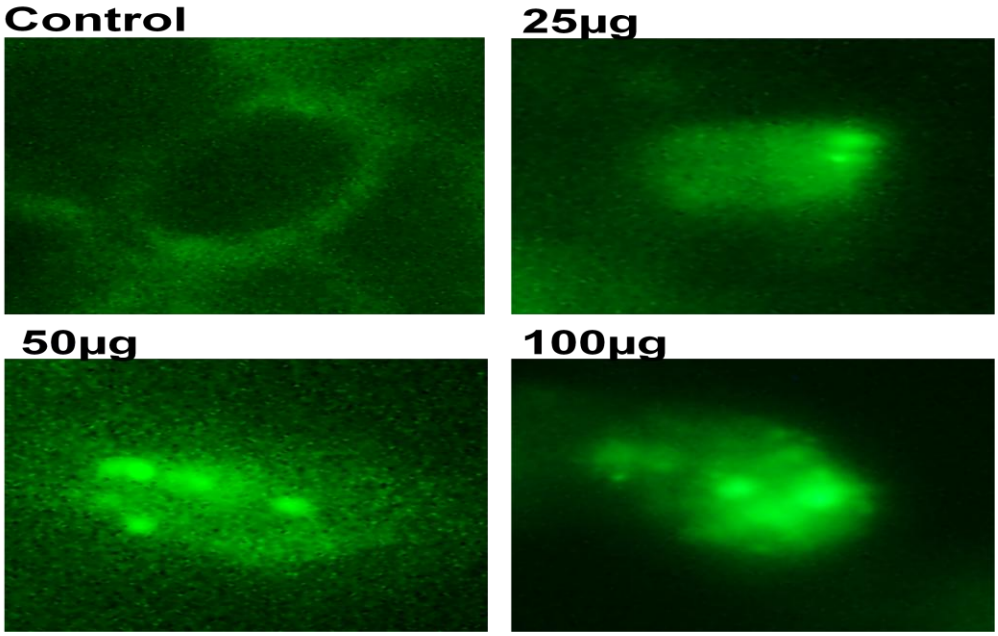

Figure-17: Images showing Puncta formation in T-98G cells with different concentration of Con A

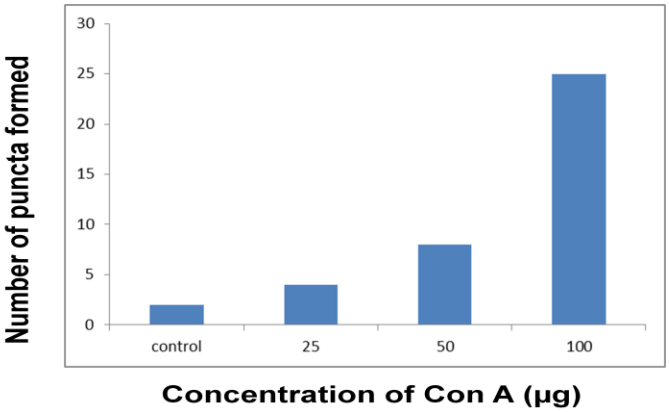

Figure-18 graph showing no. of puncta in GFP-LC3 tranfected cells

Inference:

With the increase number of puncta in a dose dependent manner, shows the increase in autophagy [figure-17]. The number of positive cell with tranfected GFP-LC3 shows puncta in dose dependent manner and more distinctly in 100µg of Con A [figure-18].

CHAPTER 7
CONCLUSION

7. CONCLUSION

This plant lectin, Con A has a very significant role, particularly to fight against the deadly glioblastoma as it contains the potency to induce two types of programmed cell death i.e. apoptosis (type I) and autophagy (type II). The DAPI staining, western blotting- PARP cleavage, Annexin V FITC staining and Caspase-3/7 Glo Assay demonstrate that Con A triggered Apoptosis in T98G cells. And from Acridine Orange and GFP-LC3 transfection study it is confirmed that Con A also induces autophagy. Hence, the study concludes that Con A induces both apoptosis and autophagy. And both apoptosis and autophagy are important as a clinical approach for the crap disease 'Cancer'. Further research should be done in order to better understand the mechanism of induction of apoptosis and autophagy by Con A and used as a clinical therapeutic agent against cancer.

CHAPTER 8

REFERENCE

8. REFERENCE:

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